Development and Characterization of an Efficient Mutant Resource of the Mega Variety, Samba Mahsuri for Rice Improvement

Gopi Potupureddi
Indian Institute of Rice Research
Vishalakshi Balija
Indian Institute of Rice Research
Suneel Ballichatla
Indian Institute of Rice Research
Gokulan CG
CCMB: Centre for Cellular and Molecular Biology CSIR
Komal Awalellu
CCMB: Centre for Cellular and Molecular Biology CSIR
Swathi Lekkala
Indian Institute of Rice Research
Karteek Jallipalli
Indian Institute of Rice Research
Ershad Mohammad
Indian Institute of Rice Research
Milton M
Indian Institute of Rice Research
Srikanth Arutla
Indian Institute of Rice Research
Rajender Burka
Indian Institute of Rice Research
Laha Shankar Gouri
Indian Institute of Rice Research
Padmakumari Phani Ayyangari
Indian Institute of Rice Research
SubbaRao Venkata Lella
Indian Institute of Rice Research
Sundaram Meenakshi Raman
Indian Institute of Rice Research
Viraktamath BC
Indian Institute of Rice Research
Ravindra Babu Vemuri
Indian Institute of Rice Research
Brahma Kranthi
CCMB: Centre for Cellular and Molecular Biology CSIR
Raju Madnala
CCMB: Centre for Cellular and Molecular Biology CSIR
Hitendra Kumar Patel
CCMB: Centre for Cellular and Molecular Biology CSIR

Ramesh Venkata Sonti
CCMB: Centre for Cellular and Molecular Biology CSIR
Maganti Sheshu Madhav (sheshu24@gmail.com)
Crop Improvement, ICAR- Indian Institute of Rice Research, Hyderabad, India https://orcid.org/0000-0001-6100-5751

Original article

Keywords: Rice, Samba Mahsuri, Mutagenesis, Mutational breeding and EMS

Posted Date: October 1st, 2020

DOI: https://doi.org/10.21203/rs.3.rs-83583/v1

Abstract

Background:

Novel variants of rice (*Oryza sativa* L.) can be obtained from induced mutations. The objective of the present study is to induce mutations in the background of Samba Mahsuri (BPT-5204), a popular and mega rice variety of India for creating novel variants for morphological, physiological and biotic stresses through using Ethyl Methane Sulphonate (EMS).

Results:

A population derived from 10, 500 M_1 plants and their descendants was phenotyped for a wide range of traits leading to the identification of 124 mutants having variations in key agro-morphological traits, and 106 mutants exhibited variation for physiological traits. Higher yield is the ultimate goal of crop improvement and we identified 574 mutants having higher yield compared to wild type by having better yield attributing traits. Further, a total of fifty mutants showed better panicle exsertion phenotypes as compared to Samba Mahsuri leading to enhancement of yield. Upon rigorous screening for three major biotic stresses, nine mutants showed enhanced tolerance for yellow stem borer, and thirteen different mutants each showed enhanced tolerance for sheath blight and bacterial leaf blight, respectively. On the whole, 1406 desired mutant lines identified at M_2 were forwarded to an advanced generation (M_5). Whole genome re-sequencing and analyses of 15 stable mutants revealed high level of similarities (99.91 to 99.99%) with the Samba Mahsuri.

Conclusions:

The lines showing enhanced tolerance to important biotic stresses (YSB, ShB and BLB) as well as several economically important traits are unique genetic resources which can be utilized for the identification of novel genes/alleles for different traits. The lines which have better agronomic features can be used as pre-breeding lines. The entire mutagenic population is maintained as a national resource for genetic improvement of the rice crop.

Background

Rice is a staple food for almost 56% of the world's population and it is the source of 20% of the world's dietary energy supply. Global rice consumption is projected to increase from 500 million to around 750 million tons by 2050 (Pathak et al. 2018). Hence, rice production has to be enhanced (50% projected growth) to match the increased requirements for the future. Development of genetic stocks suitable to the various agro-climatic zones is being done based on selection among the improved cultivars or through bringing improvements of mega varieties by modifying specific traits. This is often leading to a narrow genetic base which results in low variability and consequently limits on the possibilities for recombination and genetic segregation, leading to the reduced genetic gains (Fu, 2015). Therefore, in spite of the development and release of numerous rice varieties and hybrids, the genetic gain has been relatively low (Singh et al. 2016). Hence, there is an ample need to enhance available variation amongst the rice germplasm.

Induced mutations are one of the best available options to create variation among the well adapted mega varieties. Mutations induced artificially using physical agents like fast neutron (Ruengphayak et al. 2015), γ-ray (Lie-bao et al. 2005; Morita et al. 2009), ion beam (Lee et al. 2015) and chemical mutagens such as ethyl methane sulfonate (EMS) (Lee et al. 2003, Till et al. 2007, Mohapatra et al. 2014), methyl nitrosourea (MNU) (Suzuki et al. 2008), sodium azide (SA) (Awan et al. 1980 and Tai et al. 2016) have been employed in rice to create mutations. Chemical or physical mutagens have higher mutation efficiencies as compared to insertions created by T-DNA (Ram et al. 2019), transposable elements (Greco et al. 2001, Xuan et al. 2016) or RNAi (Wang et al. 2013), TALEN-based gene editing (Li et al. 2012), CRISPR/Cas9 genome editing (Xie et al. 2015), etc.

Among the mutagens, EMS is being used frequently owing to its production of high frequency (2 to10 mutations/ Mb) of single nucleotide changes (point mutations) by alkylation of specific nucleotides (Till et al. 2007; Viana et al. 2019) and a relatively small population, ca. 10,000 plants is sufficient to saturate the genome with mutations. According to FAO/IAEA-MVD reports (2020) (www.mvd.iaea.org), a total of 828 mutant varieties were developed in rice among them 60 were derived through chemical mutagenesis. Till date using chemical mutagens, 6 mutant resources generated in diverse *Japonica* cultivars viz. Nipponbare,

Tainung 67, Kitaano, TC65, Yukihikari, Kinmaze, and BRS Querencia and three mutant resources in *indica* (IR64, Kasalath and SSBM) and one *aus* (Nagina22) were developed (Li et al. 2017). The International Rice Functional Genomics Consortium announced the public availability of more than 200,000 rice mutant lines, which represent mutations in about half of the known functional genes mapped for rice to date (Krishnan et al. 2009). In India, one EMS mutant resource was developed in the genetic background of Nagina 22 (N22) which is a drought and heat tolerant *aus* rice variety (Mohapatra et al. 2014). This mutation resource is useful in functional genomics studies.

Samba Mashuri (BPT-5204) is a mega variety of India that was released in the year 1984 and is now also grown in other Asian countries. It is a long duration (145-150 days) fine-grain type variety, yielding an average of 5-6 t ha $^{-1}$ with excellent cooking quality and has wide adaptability. However, this variety is susceptible to biotic stresses like sheath blight, blast, bacterial leaf blight (BLB) and insect pests such as yellow stem borer (YSB), Brown plant hopper (BPH) and gall midge. Furthermore, there are varieties that yield more and are also more adapted to abiotic stresses. In spite of these constraints, it is favored by consumers and farmers in India because of its excellent cooking and eating quality characteristics. Enhancing genetic variability in the Samba Mashuri would be useful to tackle some of the existing constraints of this variety. Furthermore the excellent combining ability of this variety (Sundaram 2018) will help in transferring such traits into other varietal backgrounds. Because of the above, Samba Mashuri is an ideal genotype to develop a comprehensive mutant population.

Currently, mutagenesis coupled with molecular mapping and development of molecular markers has become an important tool with excellent potential for crop improvement (Kharkwal and Shu, 2009). Markers identified through next-generation sequencing technologies (NGS) are an efficient and cost-effective approach to characterize the variants in mutant collections (McCallum et al. 2000). Mutant resources are being used not only for creating the variation but also for the novel gene identification and deciphering gene function (Xu et al. 2017). To generate variability in the background of Samba Mahsuri, the current research focused on developing mutants using EMS and characterized them for various morphological, physiological, yield traits and biotic stress, so as to be able to use these mutants as donors in rice improvement programmes.

Materials And Methods

Mutagenesis of rice seeds

The rice variety, Samba Mahsuri (Nucleus seed obtained from Baptla Rice Research station, Andhra Pradesh, India) was used for mutagenic treatment. Based on kill curve analysis the seeds were treated in two different concentrations of Ethyl methane sulphonate (EMS) *i.e.* 1.2% (1-6819) and 0.8 % (6820-10500) by M/s Bench Biotech Company, Gujarat, India. The duration of treatment was about 12 hrs and the treated seeds were washed thoroughly under running tap water for 6 h to leach out the residual chemical.

Development of M_1 and M_2 generations

A total of 10500 M_1 plants were raised from mutagenized (M_0) seeds in the fields of M/s Bench Biotech Company, Gujarat. These M_1 plants were protected from out crossing through bagging and harvested individually to obtain the M_2 seeds from main panicles. The collected M_2 seeds were distributed among the participating centers (Centre for Cellular and Molecular Biology (CCMB) and ICAR-Indian Institute of Rice Research (ICAR-IIRR) for further studies. The M_2 seeds were raised at ICAR-IIRR and International Crop Research Institute for Semi Arid Tropics(ICRISAT), Patancheru, Hyderabad during kharif (June-July to October-November) 2013.

Phenotypic characterization of the Samba Mahsuri mutants

A large subset of 10,500 M_2 lines were subjected to various morphological (Plant height, Pink apicules, Albino, Xantha and Coloration), agronomical (panicle types, complete panicle emergence, early flowering, grain types and high yield with high grain number), physiological (strong culm, different types of flag leaf, sterile plant, stay green and shattering) and biotic stresses (sheath blight, bacterial leaf blight and yellow stem borer) studies.

Plant height was measured from the base of the plant to the tip of the tallest panicle. High yielding mutants were phenotyped based on the yield parameters like number of tillers, number of panicles, panicle length (cm), grain number per panicle and yield per plant (g) at maturity (Singh et al. 2018). The number of days from seeding to grain ripening (85% of grains on panicle are mature) was recorded and maturity duration of the mutants was observed. The lines that matured earlier to the wild type (Samba Mashuri) were selected as early maturing mutants. At the heading stage, mutants having length of > 2cm of uppermost internode were selected as EUI (Elongated Upper Internode) mutants (Xu et al. 2004). The mutants which showed complete emergence of panicle not having any choking in the flag leaf but not showing the elongated upper internode were considered as complete panicle emergence (CPE) mutants. The grain lengths were measured by using a digital Vernier caliper (M/s Mitutoyo, USA) and classified based on length and breadth of grain (Ramkumar et al. 2010). For the identification of strong culm mutants, the diameter and physical strength of the culm was taken into account. Culm diameter was measured using a Digital sliding Vernier caliper in the field from the plants at a height of 30cm above ground level (Yadav et al. 2017), while the physical strength of the culms was measured with a 'prostrate tester' (DIK-7401 Daiki Rika Kogyo co Ltd, Tokyo, Japan) where the plant was pushed at an angle of 45° to calculate the pushing resistance (Hai et al. 2005).

In each generation, the mutants with the trait of interest were identified and advanced to the next generations in ear to row method. The whole mutant population was advanced up to M_4 generation considering the diversity. Later on, from M_5 generation, the advancement of mutants was done based on yield and uniformity. The data for the above-mentioned traits were recorded at three different crop growth stages *i.e.* seedling, vegetative and reproductive stages following DUS guidelines (Mohapatra et al. 2014).

Screening of mutant lines for biotic stresses

Screening for Yellow Stem Borer (YSB) resistance/susceptibility was done in a phased manner at both vegetative and reproductive phases by augmenting the natural pest infestation through artificial releases (Devasena et al. 2018). The susceptibility and resistant level of the mutant line was determined based on the Standard Evaluation Scale (SES) scale, IRRI, 2014. The varieties, Pusa Basmati-1 and Samba Mahsuri were used as susceptible checks, while W1263 was the tolerant check for YSB screening.

Screening for sheath blight tolerance/susceptibility of the mutant population was done at two locations *viz.,* ICAR-IIRR and ICRISAT farms using a highly virulent isolate of rice sheath blight pathogen *R. solani* (WGL-12 isolate). Artificial inoculation of the isolate was done in colonized typha stem bits and culture prepared according to the procedure given by Bhaktavatsalam et al. (1978). Varieties including TN1 and Samba Mahsuri (Wild type) were used as susceptible checks, while 'Tetep' was used as a tolerant check for sheath blight screening. Observations were recorded after 20 days of inoculation and scored as per IRRI-SES scale (Standard Evaluation System) (IRRI, 1996). Similarly, the mutagenized population was evaluated for bacterial blight (BB) under artificial conditions following the clip-inoculation method (Kauffman et al 1973), the lesion length was recorded after 15 days of infection and scored according to SES scale, IRRI (2014) (Yugander et al. 2019). For BB screening, the varieties TN1 and Samba Mahsuri (Wild type) were used as susceptible checks, Improved Samba Mahsuri (a derivative of Samba Mahsuri having *Xa21,xa13* and *xa5* genes; Sundaram 2008) was used as the resistant check.

Genotyping of the mutant lines with SSR markers

Genomic similarity was carried out among the promising mutants with Samba Mahsuri using 60 SSR markers distributed across 12 chromosomes (Table S1). The DNA extraction was carried out by modified CTAB method (Saghai-Maroof et al. 1984).The PCR reaction volume was set to 15 µL and amplification was achieved using a Thermo Cycler according to the step-cycle program (Devi et al. 2015). The PCR products were run on a 3% Agarose gel stained with Ethidium bromide for 2h at 120 Volts, and DNA bands were visualized under UV light in a gel documentation system. The genomic similarity was calculated based on polymorphism between Samba Mahsuri and mutant (Table S1).

Whole genome resequencing of mutants

The genomic DNA was isolated from three genetic stocks (which include the nucleus seed of Samba Mahsuri which was used for mutagenesis along with other two stocks of this variety) as well as fifteen mutants with different traits using the CTAB method as

mentioned above. The isolated genomic DNA was checked for quality on agarose gel and quantity using Qubit 4 fluorometer (Thermo Fisher Scientific, USA). Library preparation and sequencing was outsourced to Nucleome Informatics Private Limited, Hyderabad, India. In short, sequencing libraries were prepared using Truseq Nano DNA HT Sample preparation Kit (Illumina, USA) and qualified libraries were sequenced on Illumina HiSeq2500 platform to obtain 2x150 bp reads at approximately 40X coverage.

The obtained high-quality reads from the three genetic stocks of Samba Mahsuri were used for constructing the Samba Mahsuri reference genome using the consensus calling approach described previously (Abe et al. 2012). Briefly, the sequenced reads were aligned using *bwa mem* (v0.7.17; http://bio-bwa.sourceforge.net/bwa.shtml) to the *indica* reference genome, R498 (Du et al. 2017). Using *Samtools* (v0.1.20; http://www.htslib.org/doc/samtools.html) the bam files were sorted, and deduplicated. The final bam files were used for variant calling with reference to R498 genome using *freebayes* (v1.0.0; Garrison et al. 2012). The variants were filtered using *VCF tools* (v0.1.15; Danecek et al. 2011) and bash scripts to retain only homozygous SNPs supported by a minimum of 10 reads and with minimum quality score of 30. All the SM sequences were processed in the same way and finally all the SNPs were merged to obtain a single VCF file. Using *BCF tools* (v1.7; http://samtools.github.io/bcftools/bcftools.html) *consensus*, the SNPs were replaced in the R498 reference genome to obtain SM reference genome.

The Samba Mahsuri reference genome was used for aligning the mutant sequence reads and variant calling using the same pipeline as discussed above. After obtaining the filtered SNPs, only the GC to AT type transitions were retained for further analyses. Custom scripts were used for identifying the chromosome-wise distribution of the SNPs. Dissimilarity index was calculated by dividing the total number of GC to AT type homozygous SNPs by the total number of bases in the reference genome (i.e. 390,983,850). *Tassel 5* was used for obtaining the relatedness dendrogram (Bradbury et al. 2007). SnpEff (v4.3t; Cingolani et al. 2012) was used for annotating the SNPs.

Results

Variation in yield contributing traits and biotic stress

A total of 10,500 mutants (M_1) were generated upon treatment of Samba Mahsuri with EMS mutagen in two different concentrations. Phenotypic observations at M_2 generation for various morphological, physiological and yield parameters identified 1231 mutants (~ 12% of population) showing promising variation for the traits of interest (Table 1; Table S2 to S7). These mutants were categorized for different traits, among those, the mutants showing variation in the yield contributing traits occupied the major proportion (43%), followed by physiological and panicle emergence mutants (15% each) and the panicle type and morphological trait mutants (Fig. 1). The selected mutants were subsequently advanced to M_3 and M_4 generations wherein 418 and 276 mutants were showing stable inheritance of variations respectively (Table S2 to S7). Considering the traits of interest, 180 mutants were selected and advanced up to M_5 generation for further studies (Table S2 to S7).

Upon screening of mutagenic population of M_2 for three biotic stresses (YSB, ShB and BLB), 1453 promising mutant lines were identified, of which ShB mutants occupied the major proportion (55%). These mutants were advanced to the subsequent generations and a total of 35 stable mutants were recovered in M_5 generation for the three biotic stresses (Table S8 to S10).

Variations in the morphological traits

Around (0.79%, 83 mutants) of the total population showed variation in various morphological traits (viz., tall, dwarf, grassy bonsai and pink apiculus) (Fig. 2A & 2B). Among these, mutants having tallness (130-155cm) were the highest proportion (51%, 43 mutants) of the total morphological traits, followed by dwarf mutants (43.3%; 36 mutants) which have a length <110cm. We also identified one extreme dwarf mutant (bonsai type having a shoot length of 30-45cm). This mutant had less number of internodes (3) and very narrow internodal length with grassy appearance. The details of the traits and the number of mutants observed in each generation are given in Table 1 and Table S2.

Variations in the physiological traits

The M_2 mutants were characterized for various physiological traits *viz.,* flag leaf variation, leaf variations, albino, xantha, coloration, strong culm, stay green, early maturity, shattering and sterility. Of the total population, 1.8% mutants (191) showed variations in the physiological traits. Among these, Strong culm that imparts lodging resistance is an important trait and we identified 26 mutants (0.24%) with culm strength ranging between 26-32 N/m^2, while the wild type showed culm strength of $25N/m^{2.}$ (Fig. 2C). Early maturing (108-136 days) mutants were observed at a frequency of 0.69% (73 mutants) (Fig. 2D). Stay-green in the post-anthesis period is found to be an efficient drought tolerance trait and we obtained 4 stay green mutants (0.03%) in the M_2 generation. We also obtained a good frequency (0.26%) of sterile plants; however these could not be maintained further due to non-uniform segregation (Table S3).

Variations in panicle types

Panicle types (sparse, long and dense) were studied and a proportion of 0.8% (85) of the total M_2 population was observed. Among these, long panicle (30-32cms) type mutants were the highest with 0.47% (50 mutants) (Fig. 2E). The dense panicle mutants recorded 300-350 seeds per plant, while the wild type had only 180-200 seeds (Fig. 2F; Table S4).

Variations in panicle emergence

Complete panicle emergence (CPE) and Elongated Upper Internode (EUI) are two major traits that were studied under the category of panicle emergence. In this category we identified 182 mutants (1.72% of the population) of which CPE mutants were more numerous (1.47% of the population) and the EUI mutants were less frequent (0.25% of the population). The CPE mutants exhibited 2 to 2.5cms of panicle exsertion and thus no panicle choking (Fig. 2G) whereas 10-20% panicle chocking was observed in wild type. The EUI mutants exhibited a length of 7-26cm for the upper most internode (Fig. 2H) (Table S5).

Variations in yield and yield related traits

The yield-related traits included more number of tillers, number of productive tillers and better grain filling, grain number and phenotypic acceptability (PA). In the M_2 generation, the mutants in yield traits were the most frequent; *i.e.* a total of 532 mutants representing 5.06% of the total mutant population (Fig. 2I). Among these traits, number of productive tillers and better grain filling occupied the major proportion with 4.06% (484 mutants), while more number of tillers (15-26 tillers) occupied the least with a proportion of 0.12%. Overall acceptability of the mutant lines was also recorded and we obtained a frequency of 0.19% (20 mutants) in the M_2 generation (Table S6).

Variations in the Grain types:

The wild type has medium slender grain type, however upon treatment with EMS we observed a wide range of grain types (Table 1). The grain type mutants occupied 1.5% (158) of the total M_2 population, of which medium bold grain without awns were the highest frequency (0.36%), followed by long slender grain without awns (0.34%). Interestingly, we also observed seven (0.06%) sickle shaped seed mutants. The types of variants obtained are indicated in Fig. 2J. All these mutants were forwarded to M_5 generation, however in subsequent generations we focused on medium slender and long slender grain types, as these are the commercially preferred grain types (Table S7).

Biotic stress variations among the mutants

The mutant population that had been generated was large and screening such a large population at one time for various biotic stresses in the same season was not practical. For ease of operations, 2500 M_2 families each were screened in two seasons for all three biotic stresses. While going for the second season screening, the selected tolerant mutants from the first season were carried forwarded for rescreening in the next generation along with the base mutant population. A high frequency (13.83%) of the total population showed tolerance for at least one of the three biotic stresses at M_2 generation. But rescreening was continued up to M_5 generation, the frequency of tolerance was reduced from 13.83% to 0.33%. Among those, Sheath blight tolerant mutants were the most frequent (7.62%) followed by the bacterial leaf blight tolerant (4.59%) while the YSB tolerant mutants were the least (1.61%) at the M_2 generation (Table S8 to S10). The reduction in frequency of tolerant mutants following rescreening

indicates that there were a large number of false positives possibly due to lines that escaped infection. The details of the tolerant mutants obtained in each generation for various biotic stresses are given in Table 2 and the visual symptoms are given in fig. 2K, 2L and 2M.

Mutants with multiple trait variations

Most of the mutants showed trait-specific variations in comparison with Samba Mashuri, but a few mutant lines (8) showed variation in multiple traits. For instance, the mutant line TI -24 showed different trait combinations like strong culm, early flowering, medium bold, pink apiculus, complete panicle emergence, broad flag leaf and dense panicle and stay green. Similarly, another mutant TI-21 also exhibited variations in multiple traits *viz.,* Strong culm, broad flag leaf and early flowering yet showed medium slender grain like wild type. The details of the mutants which showed multiple trait variations are given in the Table S11.

Genotyping of mutant lines:

To know the genomic similarity between mutants and wild type, a set of 60 SSR markers distributed uniformly across the genome were used. A total of 49 mutants were selected randomly for the estimation of genomic similarity, which revealed a similarity range of 85.0-98.3% in comparison to the wild type (Table S12).

We further performed whole genome resequencing of the wildtype (three genetic stocks) and fifteen mutant lines showing different traits to study the extent of mutagenesis. We obtained high quality sequences with an average depth of 41X (Table 3). Greater than 97% of the reads from the mutant lines mapped to the Samba Mahsuri reference genome (Table 3). Analyses of the EMS-induced SNPs revealed that the dissimilarity between Samba Mahsuri and the mutant lines ranges from 0.008% to 0.089% (Table 4). The number of EMS-induced homozygous SNPs in the mutant lines was between 29850 and 348872. Annotation of the SNPs showed that among the total number of SNPs, 16 to 20% of the SNPs were in the coding region of the mutant lines (Table S13) and relatedness analysis revealed clustering of the mutant lines (Figure S1).

Discussion

Samba Mahsuri (BPT-5204) is a popular rice variety in South and Eastern India which is highly susceptible to many biotic and abiotic stresses. The everlasting demand for this variety has encouraged various researchers to select this variety for marker assisted selection (MAS) to improve tolerance for some of the stresses, where the donors are available in gene pool. To enhance the scope of MAS and to develop the donors for traits where the donors are not available, creation of variation through mutagenesis was sought. Among various physical and chemical mutagens, EMS induced mutations creates more point mutations and less chromosomal segment breaks as well demonstrated in rice (Till et al 2007; Greene et al. 2003). The screening of mutants generated by T-DNA or transposons suffer from restrictions for field evaluation as they are considered as genetically modified (GM) but this restriction does not apply for the EMS induced mutants. Owing to these advantages, in the present study EMS was opted for induction of mutagenesis. Till date, more than 22 mutant resources were developed in diverse genetic backgrounds of rice using different mutagens across the globe (Sevanthi et al. 2018), but no studies were reported on the development of mutagenic population in Samba Mahsuri till date.

Identification of suitable mutagenic dose is the key component in developing mutagenic populations since it determines the mutation densities. To generate such population, Samba Mashuri seed were treated with EMS in two different concentrations (1.2% and 0.8% for 12 hrs) and the 1.2% treatment yielded a greater frequency of mutants with favored agro-morphological traits. Similar mutation induction studies were conducted by Mohapatra et al. (2014) in *aus* cultivar Nagina 22 and confirmed that 1.5% for 12 hrs of EMS treatment generated useful mutants after using a wide range of EMS concentrations (0.2-2%). Wu et al. (2005) established a mutant population in IR64 using five different EMS concentrations (0.4, 0.6, 0.8, 1.0 and 1.6%) for kill curve analysis and observed that the 1.6% concentration exhibited the high frequency rate of mutations. Talebi et al. (2012) used six different concentrations (0.25%, 0.50%, 0.75%, 1%, 1.25%, 1.5% and 2%) of EMS to treat MR219, a Malyasian *indica* variety and fixed 0.25% and 0.50% for 12 hrs as LD values for generating variation. Several mutagenesis studies on plants have reported that the optimal concentration of the chemical mutagen can vary with the variety used, which might be due to possible influence of the genome on the effects of the mutagen (Rao and Reddi, 1986).

The progeny from a set of 10,500 Samba Mahsuri M_1 mutant lines were characterized for agro-morphological traits and biotic stresses from M_2 to M_5 generations and mutants were identified that have variations in different morphological characters like height (tall, dwarf, bonsai) and pink apiculus. Similar plant height variations were also identified by Mohapatra et al. 2014. Our observations also suggested that some mutants might have mutations with large phenotypic effects which led to fall well outside the phenotype of the wild type.

The flag leaf and top leaf blades play a major role in photosynthesis and affect the grain yield of rice (Takai et al. 2013). Therefore we classified mutants having broad and long flag leaf variations in the category of physiological traits. These mutants might carry mutations among alleles/genes responsible for flag leaf development. Earlier studies demonstrated that a mutation in the Flag Leaf gene (*NAL 1*) affects various yield related traits in rice (Taguchi-Shiobara et al. 2015). Similarly, Sakamoto et al. (2005) explained that the brassino-steroid deficient mutant having erect leaves showed increase in biomass and grain yield in rice. Phenotypes such as albino, xantha and coloration mutants were observed in M_1 generation but they were not observed in adult plants of M_2 generation indicating that they were lethal.

Improvement of lodging resistance by incorporation of the semi-dwarf trait alone is not sufficient and other traits such as strong culm are needed for reinforcement of the phenotype (Ookawa et al. 2016). According to Ookawa et al.(2010), the diameter and wall thickness will influence the parameter of the physical strength of the culm. In the present study the identified 26 strong culm mutants will be used for the identification of genes/alleles that governs the trait. A QTL associated with strong culm was earlier identified by Ookawa et al. 2010. Using chromosomal segment substitution lines they have reported that *Strong Culm 2* (*SCM 2*), a gain- of -function mutant of APO1, was responsible for the strong culm phenotype. Maturity duration is one of the important growth parameters that determine crop productivity under a given climatic condition. We recovered thirty-six early maturing mutants (maturing between 108-135 days). Mutants that are similar in yield with wild type but having an early maturing nature are very useful for the rice varietal improvement programme. Similarly, Chakrabarti et al. (1995) developed early maturing PNR series mutants utilizing gamma radiations in Basmati 370 PNR-17-3 and Mustikarini et al. (2017), developed a gamma-ray induced red rice (*Celak Madu* accession) mutant which matures earlier than the wild type (*i.e.* in 115 days) and is drought tolerant .

The stay green or delayed leaf senescence phenotype of the plant is another attractive and desired characteristic of rice. Very few genetic sources are available for this trait and hence the stay green mutants identified in this study can be used in improving rice production and biomass under stressful environment conditions. In this connection it is pertinent to note that Ramkumar et al. (2019) have identified a stay green mutant (*SGM-3*) of Nagina22 which has better harvest index under drought stress.

Similarly, elongation of upper internode (EUI) is an important component of plant architecture that can be used to overcome the sheathed panicle of the rice male sterile line (Shen et al. 1987) by enhancing pollen dispersal and facilitating hybrid rice seed production (Yang et al. 2002). In our study, the identified EUI mutants were observed to have better upper node elongation than the wild type and their incorporation into the CMS line can help enhance pollen dispersal. Okuno and Kawai (1978) first reported EUI mutants, derived from the Japanese rice cultivar Norin 8 by gamma-ray treatment. Similar EUI studies were conducted by Rutger and Carnahan (1981) and reported enhanced upper internode elongation during the heading stage. Similarly, incomplete panicle emergence leads to yield reduction (Yuan et al. 1988) which is generally observed in Samba Mahsuri resulting in 10-15% reduction in yield. Hence, the identified mutants that have complete panicle emergence may play an important role in rice breeding programmes to increase yield of Samba Mahsuri as well as other rice varieties whose yield may be affected due to incomplete panicle emergence.

A number of high yielding mutant lines were identified that have more tillers number and high grain number. Similarly, Roy et al. (2018) identified eight high yielding aromatic mutants in the background of Tulpanji using gamma irradiation. Soomro et al. (2002) identified two high yielding mutant lines derived from of IR6, through gamma rays. Several high yielding mutants were also popular in many countries such as Vietnam (55 mutant varieties), Bangladesh (44 mutant varieties) and Thailand (two aromatic mutant varieties (RD6, RD15) where they have been cultivated over millions of hectares (Oladosu et al. 2016). In India, the PNR-381 and PNR-102 aromatic rice varieties are early maturing rice mutants that are derived from the Basmati 370 PNR-17-3 cultivar and have become popular in Haryana and Uttar Pradesh States (https://mvd.iaea.org; Chakrabarti, 1995).

In the present study, different grain type mutants were isolated. They could have importance in varietal improvement since the visual characteristics of rice grains is an important attribute that affects consumer's preference. Azad et al. (2012) developed different grain type mutants by irradiating IR8 with gamma rays. Different grain types (short, long, bold) were also reported from the Nagina22 mutant population (Mohapatra et al. 2014). A mutant line derived from Kitake rice variety using fast-neutron (FN) mutagenesis carried a new *grain shape 9–1* (*gs9–1*) allele that also affects grain size (Jiang et al. 2019).

In this study, mutants having variations in multiple traits were observed and such kinds of mutants were also reported earlier in Nagina 22 background (Mohapatra et al. 2014). These mutants may either carry point mutations in multiple genes that independently affect the observed traits or carry a mutation that has a pleiotropic phenotype (Patil, 1966; Till et al. 2007).

In the present study mutation frequency of many mutant types showed a decreasing in the number as we progressed from M_2 to M_5. We are unable to explain this apparent reversion phenomenon as the phenotype was quite apparent in the M_2 generation. One possibility is that there are some physiological changes, such as epigenetic changes, that arise due to mutagenesis and which revert back to the wild type phenotype/physiological state in subsequent generations. Brooks et al. 2008 reported that rice bran gene mutation restoration from white rice pericarp to red like wild type. In our study, one example of such a reversion was the sickle shape seed phenotype in M_2 generation which had reverted back to wild type seed shape by the M5 generation.

Biotic stresses (insect pests and diseases) cause more than 42% of crop yield reduction in the world (Pimentel, 1997). Among the biotic stresses, Yellow stem borer, Sheath blight and BLB count amongst important biotic stresses in rice. The resistance breeding for yellow stem borer has not gained any momentum due to the lack of suitable resistance/tolerance sources (Makkar and Bentur, 2017). Sheath Blight is another challenging disease due to unavailability of a reliable resistance source (Chen et al. 2019). Whereas, for management of BB, there is an emergent need to widen the repertoire of resistance genes to enhance the spectrum and durability of resistance to *Xanthomonas. oryzae*. Mutagenesis in a gene may result in loss or gain in function with varying levels of expression and such type of mutations have prime importance while dealing with biotic stresses. Therefore, we attempted to identify resistance/tolerance stocks for YSB, Sheath blight and BB from the mutagenized population. In a similar mutation study conducted by Wu et al. (2005), by irradiating IR64 with gamma rays, rice mutants that are resistant to Blast, BB and tungro disease were identified. Similarly, Mohapatra et al. (2014) identified BB resistance mutants in an EMS induced Nagina 22 population. Our study is consistent with the above mentioned studies which indicate that mutagenized populations harbor a great amount of variability that can be recovered if the mutant population is subjected to appropriate screening techniques.

We have also evaluated the similarity of the mutant lines in comparison to the wild type line using a set of 60 SSR markers that are considered to be very polymorphic and which are fairly well distributed throughout the genome as well as by resequencing. SSR analysis, the mutants exhibit a wide range of diversity ranging from about 1% to 14% in comparison to the wild type line. Earlier studies reported that the average SSR marker polymorphism between closely related cultivars was 12-15% due to conserved nature of SSR markers (Shanmugavadivel et al. 2013; Yoon et al. 2006; Govindaraj et al. 2005). Genomic similarity analysis with SSRs may depend on the selection markers and the genome wide distribution of markers. However, whole genome analyses of mutants gave the clear picture of diversity which ranged from 0.008% to 0.089%. These results indicated that generated mutants were similar to the wild type line.

Recent advances in high throughput sequencing technologies and computational approaches have facilitated the mapping of causal mutations in mutant lines (Austin et al. 2011; Schneeberger, 2014). In addition to gene identification, an allelic series comprised of INDELs can be useful in assessing gene function and for practical purposes of crop improvement. Therefore, comparison among the allelic series in SNP and INDELs across mutants and natural germplasm helps to infer the functional polymorphism as related to phenotypes observed in mutants and germplasm.

The mutants identified in this screen, especially those that have agronomically important phenotypes with significant similarity to the wild type could be useful to use in MutMap and QTL Seq for the identification of linked SNPs. The SNPs that are linked to the phenotype can be readily used in introgression programmes. Such studies will generate new knowledge and also have the potential for application in the crop improvement.

Conclusion

Exploiting genetic variability induced through mutations can be a powerful strategy in rice improvement where genetic gains may be diminishing due to a narrow gene pool. To generate variability, we have produced a large Samba Mahsuri mutant population that can be further employed in crop improvement. The huge demand for this variety and its wider adaptability and combining ability make this mutant population a very useful resource for rice improvement. The value of these stocks will be enhanced with increasing usage and extensive screening under a wide range of conditions. Systematic screens and mapping studies will not only help in generating new knowledge but will also help in translating the knowledge for crop improvement.

Abbreviations

SSR: Simple Sequence Repeats

EMS: Ethyl Methane Sulphonate

SNP: Single Nucleotide Polymorphism

INDELS: Insertions and deletions

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

Not applicable

Competing Interests

The authors declare that they have no competing interests.

Funding

Not applicable

Author's contribution:

RVS and MSM designed all experiments, developed the structure and arguments for the paper, made critical revisions, and approved the final version. PG, BV, BS and LS carried out the major work and prepared manuscript and contributed to the refinement of the manuscript. CGG, AK, KB, RM and HKP carried out whole genome sequencing and sequence analysis. JK and APP carried out the YSB screening. ME, GSL, MM, AS and BR helped in the screening of sheath blight and bacterial blight. LV, BCV and RV helped in the phenotypic evaluation of agro-morphological traits. RMS helped in molecular work. All authors have read and approved the final manuscript.

Acknowledgements

The authors acknowledge the Council of Scientific and Industrial Research (CSIR), New Delhi, India and ICAR -IIRR for providing funds and infrastructure for carrying out the research work. RVS is supported by a J C Bose Fellowship from the Science and Engineering Research Board, Government of India.

Author's information

P. Gopi, Email: gopibiotech.1985@gmail.com

B. Vishalakshi, Email: vishalakshi.biotech@gmail.com

B. Suneel, Email: suneel.biotechnology@gmail.com

CG. Gokulan, Email: gokulan@ccmb.res.in

A. Komal, Email: komal@ccmb.res.in

L. Swathi, Email: swathi.lvs@gmail.com

J. Karteek, Email: jallipalli.karteek@gmail.com

Md. Ershad, Email: ershad.cool@gmail.com

M. Milton, Email: milton2526@live.com

A. Srikanth, Email: srikanth.arutla977@gmail.com

B. Rajender, Email: rajender.burka007@gmail.com

GS. Laha, Email: lahags@yahoo.co.in

AP. Padmakumari, Email: padmakumariento@gmail.com

LV. Subbarao, Email: lvsubbarao1990@gmail.com

RM. Sundaram, Email: rms_28@rediffmail.com

B.C. Viraktamath, Email: mathrice@gmail.com

V. Ravindra Babu, Email: rbvemuri1955@gmail.com

B. Kranthi, Email: kranthi.brahma@ccmb.res.in

M. Raju, Email: raju.m@ccmb.res.in

HK. Patel Email: hitendrakpatel@gmail.com; hkpatel@ccmb.res.in

RV. Sonti, Email: sonti@ccmb.res.in

M. Sheshu Madhav, Email: sheshu24@gmail.com; sheshu_24@yahoo.com

References

1. Abe A, Kosugi S, Yoshida K, Natsume S, Takagi H, Kanzaki H, Matsumura H, Yoshida K, Mitsuoka C, Tamiru M, Innan H (2012) Genome sequencing reveals agronomically important loci in rice using MutMap. Nat biotechnol 30:174-178. doi: 10.1038/nbt.2095.
2. Austin RS, Vidaurre D, Stamatiou G, Breit R, Provart NJ, Bonetta D, Zhang J, Fung P, Gong Y, Wang PW, McCourt P (2011) Next-generation mapping of Arabidopsis genes. The Plant J 67:715-25. doi: 10.1111/j.1365-313X.2011.04619.x.
3. Awan MA, Konzak CF, Rutger JN, Nilan RA (1980) Mutagenic effects of sodium azide in rice. Crop Sci 20:663-668. doi: 10.2135/cropsci1980.0011183X002000050030x
4. Azad MAK, Uddin MI, Azam MA (2012) Achievements in Rice research at BINA through Induced mutation. Global Science Books: Bioremediation, Biodiversity and Bioavailability 6:53-57.
5. Bhaktavatsalam G, Satyanarayana K, Reddy APK, John VT (1978) Evaluation for sheath blight resistance in rice. Int Rice Res Newsletter 3:9-10.
6. Bradbury PJ, Zhang Z, Kroon DE, Casstevens TM, Ramdoss Y, Buckler ES (2007) TASSEL: software for association mapping of complex traits in diverse samples. Bioinformatics 23:2633-2635. doi: 10.1093/bioinformatics/btm308.
7. Brooks SA, Yan W, Jackson AK, Deren CW (2008) A natural mutation in rc reverts white-rice-pericarp to red and results in a new, dominant, wild-type allele: *Rc-g*. Theor Appl Genet 117:575-80. doi: 10.1007/s00122-008-0801-8.

8. Chakrabarti SN (1995) Mutation breeding in India with particular reference to PNR rice varieties. J Nucl Agric Biol 24:73-82.
9. Chen Z, Feng Z, Kang H, Zhao J, Chen T, Li Q, Gong H, Zhang Y, Chen X, Pan X, Liu W (2019) Identification of New Resistance Loci Against Sheath Blight Disease in Rice Through Genome-Wide Association Study. Rice Sci 26:21-31. doi: 10.1016/j.rsci.2018.12.002
10. Cingolani P, Platts A, Wang LL, Coon M, Nguyen T, Wang L, Land SJ, Lu X, Ruden DM (2012) A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. Fly 6:80-92. doi: 10.4161/fly.19695
11. Danecek P, Auton A, Abecasis G, Albers CA, Banks E, DePristo MA, Handsaker RE, Lunter G, Marth GT, Sherry ST, McVean G (2011) The variant call format and VCFtools. Bioinformatics 27:2156-2158. doi: 10.1093/bioinformatics/btr330
12. Dellaporta SL, Wood J and Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep 1:19-21.
13. Devasena N, Soundararajan RP, Reuolin SJ, Jeyaprakash P, Robin S (2018) Evaluation of Rice Genotypes for Resistance to Yellow Stem borer, Scirpophaga incertulas (Walker) through Artificial Screening Methods. JEZS 6:874-87.
14. Devi SJSR, Singh K, Umakanth B, Vishalakshi B, Renuka P, Sudhakar KV, Prasad MS, Viraktamath BC, Babu VR, Madhav MS (2015) Development and Identification of Novel Rice Blast Resistant Sources and Their Characterization Using Molecular Markers. Rice Sci 22:300–308. doi: 10.1016/j.rsci.2015.11.002
15. Du H, Yu Y, Ma Y, Gao Q, Cao Y, Chen Z, Ma B, Qi M, Li Y, Zhao X, Wang J (2017) Sequencing and de novo assembly of a near complete indica rice genome. Nat commun 8:1-12. doi: 10.1038/ncomms15324
16. Fu Y (2015) Understanding crop genetic diversity under modern plant breeding. Theor Appl Genet 128:2131–2142. doi: 10.1007/s00122-015-2585-y.
17. Garrison E, Marth G (2012) Haplotype-based variant detection from short-read sequencing. *arXiv*, arXiv:1207.3907
18. Govindaraj P, Arumugachamy S, Maheswaran M (2009) Bulked segregant analysis to detect main effect QTL associated with grain quality parameters in Basmati 370/ASD 16 cross in rice *Oryza sativa* L) using SSR markers. Euphytica 144:61–68. doi: 10.1007/s10681-005-4316-y
19. Greco R, Ouwerkerk PBF, Sallaud C, Kohli A, Colombo L, Puigdomenech P, Guiderdoni E, Christou P, Hoge JHC, Pereira A (2001) Transposon insertional mutagenesis in rice. Plant Physiol 125:1175–1177. doi: 10.1104/pp.125.3.1175
20. Greene EA, Codomo CA, Taylor NE, Henikoff JG, Till BJ, Reynolds SH, Enns LC, Burtner C, Johnson JE, Odden AR, Comai L (2003) Spectrum of chemically induced mutations from a large-scale reverse-genetic screen in Arabidopsis. Genetics 64:731-740.
21. Hai L, Guo H, Xiao S, Jiang G, Zhang X, Yan C, Xin Z, Jia J (2005) Quantitative trait loci (QTL) of stem strength and related traits in a doubled-haploid population of wheat (Triticum aestivum L.). Euphytica141:1-9. doi: 10.1007/s10681-005-4713-2
22. IRRI (2014), Standard Evaluation System for Rice. International Rice Research Institute, Manila, the Philippines
23. IRRI, (1996) **Standard Evaluation System for Rice** (4th edn), International Rice Research Institute, Manila, the Philippines
24. Jiang L, Li G, Chern M, Jain R, Pham NT, Martin JA, Schackwitz WS, Zhao J, Ruan D, Huang R, Zheng J (2019) Whole-Genome Sequencing Identifies a Rice Grain Shape Mutant, gs9–1. Rice 12:52. doi: 10.1186/s12284-019-0308-8
25. Kauffman, H E, Reddy, A P K, Hsieh, S PY, and Merca, SD (1973). An improved technique for evaluating resistance of rice varieties to Xanthomonas oryzae. Plant Dis. Rep. 57, 537–541.
26. Kharkwal MC, Shu QY (2009) The role of induced mutations in world food security. Shu QY (eds) In: Induced plant mutations in the genomics era. Food and Agriculture Organization of the United Nations, Rome, p 33–38
27. Krishnan A, Guiderdoni E, An G, Hsing YI, Han CD, Lee MC, Yu S, Upadhyaya N, Ramachandran S, Zhang Q, Sundaresan V, Hirochika H, Leung H, Pereira A (2009) Mutant resources in rice for functional genomics of the grasses. Plant Physiol 149:165–170. doi: 10.1104/pp.108.128918
28. Lee J, Lee AR, Kwon SW (2015) Effect of proton beam irradiation on M1 seeds and seedling growth in rice. Plant Breeding and Biotechnology 3:384–388. 10.9787/PBB.2015.3.4.384
29. Lee SY, Cheong JI, Kim TS (2003) Production of double haploids through anther culture of M1 rice plants derived from mutagenesis fertilized egg cell. Plant Cell Rep 22:218-223. doi: 10.1007/s00299-003-0663-0

30. Li GR, Jain M, Chern NT, Pham JA, Martin T, Wei WS, Schackwitz AM, Lipzen PQ, Duong KS, Jones L, Jiang D, Ruan D, Bauer Y, Peng KW, Barry J, Schmutz and Ronald PC (2017) The sequences of 1504 mutants in the model rice variety kitaake facilitate rapid functional genomic studies. Plant Cell 29:1218-1231. doi: 10.1105/tpc.17.00154
31. Li T, Liu B, Spalding MH, Weeks DP, Yang B (2012) High-efficiency TALEN-based gene editing produces disease-resistant rice. Nat Biotechnol 30:390-392. doi: 10.1038/nbt.2199
32. Lie-bao WYH, Shu-xia Y, Jin-lai W, Xiao-xia C (2005) the effect of γ-rays irradiation on seeds germination and enzyme activity of tall fescue. Journal of Nuclear Agricultural Sciences:3
33. Makkar GS, Bentur JS (2017) Breeding for Stem Borer and Gall Midge Resistance in Rice. In: R. Arora, S. Sandhu (eds) Breeding Insect Resistant Crops for Sustainable Agriculture. Springer, Singapore, pp 323-352. doi: 10.1007/978-981-10-6056-4_11
34. McCallum CM, Comai L, Greene EA, Henikoff S (2000) Targeting Induced Local Lesions in Genomes (TILLING) for plant functional genomics. Plant Physiol 123:439–442. doi: 10.1104/pp.123.2.439
35. Mohapatra T, Robin S, Sarla N, Sheshashayee M, Singh AK, Singh K, Singh NK, Amitha Mithra SV, Sharma RP (2014) EMS induced mutants of upland rice variety Nagina22: generation and characterization. Proc Indian Natl Sci Acad 80:163-172. doi: 10.16943/ptinsa/2014/v80i1/55094
36. Morita R, Kusaba M, Iida S, Yamaguchi H, Nishio T, Nishimura M (2009) Molecular characterization of mutations induced by gamma irradiation in rice. Genes Genet Syst 84: 361–370. doi: 10.1266/ggs.84.361
37. Mustikarini ED, Ardiarini NR, Basuki N and Kuswanto K (2016) The improvement of early maturity red rice mutant trait for drought tolerance. International Journal of Plant Biology, 7:48-52. doi: 10.4081/pb.2016.6345
38. Okuno K, Kawai T (1978) Variations of internode length and other characters in induced long-culm mutants of rice. Jpn J Breed 28:243-250. doi: 10.1270/jsbbs1951.28.243
39. Ookawa T, Aoba R, Yamamoto T, Ueda T, Takai T, Fukuoka S, Ando T, Adachi S, Matsuoka M, Ebitani T and Kato Y (2016) Precise estimation of genomic regions controlling lodging resistance using a set of reciprocal chromosome segment substitution lines in rice. Sci Rep 6:30572. doi: 10.1038/srep30572
40. Oladosu Y, Rafii MY, Abdullah N, Hussin G, Ramli A, Rahim HA, Miah G, Usman M. (2016) Principle and application of plant mutagenesis in crop improvement: a review. Biotechnology & Biotechnological Equipment 30:1-16, doi: 10.1080/13102818.2015.1087333
41. Pathak H, Nayak AK, Jena M, Singh ON, Samal P and Sharma SG (2018) Rice Research for Enhancing Productivity, Profitability and Climatic Resilience. Cuttack: ICAR - National Rice Research Institute.
42. Patil SH (1966) Mutations induced in groundnut by X-rays. Indian J Gent Pl Breed 26: 334-348.
43. Pimentel D (1997) Pest management in agriculture. In Pimentel D (Ed) Techniques for Reducing Pesticide Use: Economic and Environmental Benefits. Chichester, United Kingdom: Wiley, pp 1–11.
44. Ram H, Soni P , Salvi P, Gandass N , Sharma A, Kaur A, Sharma TR (2019) Insertional Mutagenesis Approaches and Their Use in Rice for Functional Genomics. Plants. 8:310. doi: 10.3390/plants8090310
45. Ramkumar G, Sivaranjani AKP, Pandey MK, Sakthivel K, Shobha Rani N, Sudarshan I, Prasad GSV, Neeraja CN, Sundaram RM, Viraktamath BC, Madhav MS (2010) Development of a PCR-based SNP marker system for effective selection of kernel length and kernel elongation in rice. Mol Breeding 26:735–740. doi: 10.1007/s11032-010-9492-3
46. Ramkumar MK, Senthil Kumar S, Gaikwad K, Pandey R, Chinnusamy V, Singh NK, Singh AK, Mohapatra T, Sevanthi AM (2019) A novel stay-green mutant of rice with delayed leaf senescence and better harvest index confers drought tolerance. Plants 8:375. doi: 10.3390/plants8100375
47. Rao DRM, Reddi TS (1986) Azide mutagenesis in rice. Proceedings: Plant Sciences. 96: 205-215.
48. Roy B, Kumar V, Tulsiram SD, Das BK (2018) Development of high yielding aromatic mutants of rice (Oryza sativa L.) from a local aromatic cultivar, Tulaipanji by using gama radiation. Indian J Genet 78:409-416. doi: 10.31742/IJGPB.78.4.2
49. Rutger, JN and Carnahan, HL (1981) A fourth genetic element to facilitate hybrid cereal production-A recessive tall in rice. Crop Sci 21 373–376. doi: 10.2135/cropsci1981.0011183X002100030005x

50. Ruengphayak S, Ruanjaichon V, Saensuk C, Phromphan S, Tragoonrung S, Kongkachuichai R, Vanavichit A (2015) Forward screening for seedling tolerance to Fe toxicity reveals a polymorphic mutation in ferric chelate reductase in rice. Rice 8:3. doi: 10.1186/s12284-014-0036-z
51. Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW (1984) Ribosomal DNAspacer-length polymorphisms in barley: Mendelian inheritance, chromosomallocation, and population dynamics. Proc Natl Acad Sci U S A 81:8014–8018
52. Sakamoto T, Morinaka Y, Ohnishi T, Sunohara H, Fujioka S, Ueguchi-Tanaka M, Mizutani M, Sakata K, Takatsuto S, Yoshida S, Tanaka H (2006) Erect leaves caused by brassinosteroid deficiency increase biomass production and grain yield in rice. Nat Biotechnol 24:105-109. doi: 10.1038/nbt1173
53. Schneeberger K (2014). Using next-generation sequencing to isolate mutant genes from forward genetic screens. Nat Rev Genet 15:662-676. doi: 10.1038/nrg3745
54. Sevanthi A, Kandwal P, Kale PB, Prakash C, Ramkumar MK, Yadav N, Mahato AK, Sureshkumar V, Behera M, Deshmukh RK, Jeyaparakash P (2018) Whole genome characterization of a few EMS-induced mutants of upland rice variety Nagina 22 reveals a staggeringly high frequency of SNPs which show high phenotypic plasticity towards the wild-type. Front Plant Sci 4:1179. doi: 10.3389/fpls.2018.01179
55. Shanmugavadivel PS, Amitha Mithra SV, Dokku P, Anand Raj Kumar K, Rao GJN (2013) Mapping quantitative trait loci (QTL) for grain size in rice using a RIL population from Basmati × indica cross showing high segregation distortion. Euphytica 194:401–416. doi: 10.1007/s10681-013-0964-5
56. Shen ZT (1987). Studies on eliminating panicle enclosure in WA type MS line of rice Oryza sativa subsp. indica. Chin J Rice Sci 1:95-99.
57. Singh N, Choudhury DR, Tiwari G, Singh AK, Kumar S, Srinivasan K, Tyagi RK, Sharma AD, Singh NK, and Singh R (2016) Genetic diversity trend in Indian rice varieties: an analysis using SSR markers. BMC Genet 17: 127. doi: 10.1186/s12863-016-0437-7
58. Singh VK, Ellur RK, Singh AK, Nagarajan M, Singh BD, Singh NK (2018) Effect of qGN4.1 QTL for grain number per panicle in genetic backgrounds of twelve different mega varieties of rice. Rice 11:8. doi: 10.1186/s12284-017-0195-9
59. Sundaram RM, Vishnupriya MR, Biradar SK, Laha GS, Reddy AG, Rani NS, Sarma NP, Sonti RV (2008) Marker assisted Marker assisted introgression of bacterial blight resistance in Samba Mahsuri, an elite indica rice variety. Euphytica 160:411–422
60. Sundaram RM, Madhav MS, Neeraja CN, Balachandran SM, Mangrauthia SK, Barbadikar KB, Divya PS, Rao LV Subba, Hariprasad AS (2018). Multi-trait improvement in rice through marker-assisted breeding ORYZA- 55: 24-37
61. Soomro AM, Baloch AW, Ahmed M, Javed MA, Bughio HR, Bughio MS, Muhammad T, Mastoi NN (2002) Evaluation of Induced Mutants of Rice for Yield and Quality Characters. Asian Journal of Plant sciences 3:248-249. doi: 10.3923/ajps.2002.248.249
62. Suzuki T, Eiguchi M, Kumamaru T, Satoh H, Matsusaka H, Moriguchi K, Nagato Y, Kurata N (2008). MNU-induced mutant pools and high performance TILLING enable finding of any gene mutation in rice. Mol Genet Genom 279:213–223. doi: 10.1007/s00438-007-0293-2
63. Taguchi-Shiobara F, Ota T, Ebana K, Ookawa T, Yamasaki M, Tanabata T, Yamanouchi U, Wu J, Ono N, Nonoue Y, Nagata K (2015) Natural Variation in the Flag Leaf Morphology of Rice Due to a Mutation of the NARROW LEAF 1 Gene in Oryza sativa L. Genetics 201:795-808. doi: 10.1534/genetics.115.181040
64. Tai TH, Chun A, Henry IM , Ngo KJ , Burkart-Waco D (2016) Effectiveness of Sodium Azide Alone Compared to Sodium Azide in Combination with Methyl Nitrosurea for Rice Mutagenesis. Plant Breeding and Biotechology 4:453-461. doi: 10.9787/PBB.2016.4.4.453
65. Takai T, Adachi S, Taguchi-Shiobara F, Sanoh-Arai Y, Iwasawa N, Yoshinaga S, Hirose S, Taniguchi Y, Yamanouchi U, Wu J, Matsumoto T (2013) A natural variant of NAL1, selected in high-yield rice breeding programs, pleiotropically increases photosynthesis rate. Sci Rep 29:2149. doi: 10.1038/srep02149
66. Talebi AB, Talebi AB, Shahrokhifar B (2012) Ethyl methane sulphonate (EMS) induced mutagenesis in Malaysian rice (cv. MR219) for lethal dose determination. American Journal of Plant Sciences: 3:1661. doi: 10.4236/ajps.2012.312202

67. Till BJ, Cooper J, Tai TH, Colowit P, Greene EA, Henikoff S, Comai L (2007) Discovery of chemically induced mutations in rice by TILLING. BMC Plant Biol 7:19. doi: 10.1186/1471-2229-7-19
68. Viana VE, Pegoraro C, Busanello C and de Oliveira AC (2019) Mutagenesis in Rice: The Basis for Breeding a New Super Plant. Front Plant Sci 10:1326. doi: 10.3389/fpls.2019.01326
69. Wang N, Long T, Yao W, Xiong L, Zhang Q, Wu C (2013) Mutant Resources for the Functional Analysis of the Rice Genome. Mol Plant 6:596-604. doi: 10.1093/mp/sss142
70. Wu JL, Wu C, Lei C, Baraoidan M, Bordeos A, Madamba MRS, Ramos-Pamplona M, Mauleon R, Portugal A, Ulat VJ and Bruskiewich R (2005) Chemical-and irradiation-induced mutants of indica rice IR64 for forward and reverse genetics. Plant Mol Biol 59:85-97. doi: 10.1007/s11103-004-5112-0
71. Xie K, Minkenberg B, and Yang Y (2015) Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. Proc Natl Acad Sci USA 112:3570–3575. doi: 10.1073/pnas.1420294112
72. Xu R, Qin R, Li H, Li D, Li L, Wei P, Yang J (2017) Generation of targeted mutant rice using a CRISPR-Cpf1 system. Plant Biotechnol J 15: 713–717. doi: 10.1111/pbi.12669
73. Xu Y, Zhu Y, Zhou H, Li Q, Sun Z, Liu Y, Lin H, He Z (2004) Identification of a 98-kb DNA segment containing the rice *Eui* gene controlling uppermost internode elongation, and construction of a TAC transgene sublibrary. Mol Genet Genomics 272:149–155. doi: 10.1007/s00438-004-1019-3
74. Xuan YH, Kim CM, Je B, Liu JM, Li TY, Lee G-S, Kim T-H, Han C-D (2016) Transposon Ds -mediated insertional mutagenesis in rice (Oryza sativa). Curr Protoc Plant Biol 1:466–487. doi: 10.1002/cppb.20030
75. Yadav S, Singh UM, Naik MS, Venkateshwarlu C, Ramayya P, Raman A, Sandhu N, Kumar A (2017) Molecular mapping of QTLs associated with lodging resistance in dry direct-seeded rice (Oryza sativa L.). Front Plant Sci 8:1431. doi: 10.3389/fpls.2017.01431
76. Yang RC, Zhang SB, Huang RH, Yang SL and Zhang QQ (2002) Breeding technology of eui-hybrids of rice. Agricultural Sciences in China 1:359-363.
77. Yoon D-B, Kang KH, Kim HJ, Ju HG, Kwon SJ, Suh JP, Jeong OY, Ahn SN (2006) Mapping quantitative trait loci for yield components and morphological traits in an advanced backcross population between Oryza grandiglumis and the O. sativa japonica cultivar Hwaseongbyeo. Theor Appl Genet. 112:1052-62. doi: 10.1007/s00122-006-0207-4
78. Yuan LP, Virmani SS (1988) Status of hybrid rice research and development. Hybrid Rice IRRI: 7-24.
79. Yugander A, Sundaram RM, Singh K, Ladhalakshmi D, Rao LVS, Madhav MS, Badri J, Prasad MS, Laha GS (2018) Incorporation of the novel bacterial blight resistance gene Xa38 into the genetic background of elite rice variety improved Samba Mahsuri. PLoS One 13:e0198260. doi: 10.1371/journal.pone.0198260

Tables

Table 1: Number of mutants that exhibit agronomically important traits in each generation from M_2 to M_5 generation

No.	Character	M_2	M_3	M_4	M_5
I	Morphological Characters				
	Tall plant	43	12	10	7
	Dwarf	36	4	2	1
	Bonsai (grassy)	1	1	1	1
	Pink apicules	3	3	1	1
	Total (I)	**83**	**20**	**14**	**10**
II.	**Physiological Characters**				
	Flag leaf variations (Broad & Long)	28	18	15	9
	Leaf variations (Narrow)	7	4	4	1
	Albino	5	-	-	-
	Xantha	5	-	-	-
	Coloration	11	-	-	-
	Strong culm	26	17	17	17
	Maturity (Early flowering)	73	36	36	36
	Stay green	4	3	2	2
	Sterile plant	28	-	-	-
	Shattering	4	3	2	2
	Total (II)	**191**	**81**	**76**	**67**
III.	**Panicle Type**				
	Sparse Panicle	5	3	2	2
	Compact/Dense Panicle	30	13	8	5
	Long Panicle	50	32	28	15
	Total (III)	**85**	**48**	**38**	**22**
IV.	**Panicle Emergence**				
	Elongation of Upper Internode (EUI)	27	11	11	9
	Complete panicle emergence	155	31	22	13
	Total (IV)	**182**	**42**	**33**	**22**
V.	**Yield**				
	More number of tillers	13	9	8	5
	More productive tillers & better grain filling	484	120	22	8
	High grain number	15	3	2	2
	Phenotypic acceptability	20	8	4	2
	Total (V)	**532**	**140**	**36**	**17**
VI.	**Grain types**				
	Long slender grain without awns	32	18	18	7

	Long slender grain with awns	11	11	10	3
	Long bold grain with awns	2	2	1	1
	Long bold grain without awns	27	16	14	10
	Medium slender grain with awns	4	4	3	1
	Medium bold grain with awns	2	2	1	1
	Medium bold grain without awns	38	22	20	15
	Short bold	13	4	4	2
	Short slender	22	5	5	2
x.	Sickle shape	7	3	3	-
	Total (VI)	**158**	**87**	**79**	**42**
	Total (I+II+III+IV+V+VI)	**1231**	**418**	**276**	**180**

Table 2: Number of mutants that exhibit resistance against YSB, BLB and Sheath Blight from M_2 to M_5 generations

S. No	Trait	M_2	M_3	M_4	M_5
1.	Yellow Stem Borer (YSB)	170	30	10	09
2.	Bacterial Leaf Blight (BLB)	482	261	40	13
3.	Sheath Blight (SB)	801	620	40	13
	Total				**35**

Table 3: Sequence statistics of Samba Mahsuri (wild type) and mutant lines with different traits

S.No.	Line	Type or trait of Line	Number of Raw Reads (R1+R2)	QC Passed Reads (R1+R2)	Sequenced bases (Gb)	Coverage (X)	Mapped QC passed Reads (R1+R2)	Mapped Reads (%)	Reference used
1	SM-E	Wildtype	112285084	102241208	15.34	39.22	100885182	98.67	R498
2	SM-G	Wildtype	110937222	111916395	16.79	42.94	110657624	98.88	
3	SM-C	Wildtype	123573016	111003694	16.65	42.59	122513940	98.27	
4	TI-109	Complete Panicle Exsertion	114514030	114191395	17.13	43.81	103577844	98.73	Samba Mahsuri
5	TI-110	Complete Panicle Exsertion	107814208	96892528	14.53	37.17	96892528	98.77	
6	TI-128	High Yield; High Spikelets per Panicle	113249162	102101092	15.32	39.17	102101092	98.89	
7	TI-140	Long Slender Grain	113457634	113352478	17.00	43.49	101932521	99.02	
8	TI-17	Sheath Blight Tolerance and Strong Culm	183078746	157972948	23.70	60.61	156353807	98.98	
9	TI-170B	Strong Culm	111938794	100095802	15.01	38.40	100095802	98.55	
10	TI-26	Robust Growth and Strong Culm	125540188	109172828	16.38	41.88	106334494	97.40	
11	TI-35	Elongated Uppermost Internode	108635160	108364345	16.25	41.57	97603326	98.76	
12	TI-38	Complete Panicle Exsertion	113152500	102614244	15.39	39.37	102614244	98.86	
13	TI-42	Bacterial Blight Tolerance	106403198	78348650	11.75	30.06	78348650	98.62	
14	TI-50	High Yield; High Spikelets per Panicle	109924526	99290257	14.89	38.09	99290257	98.90	
15	TI-51	Long Slender Grain	112746444	101548559	15.23	38.96	101548559	98.62	
16	SB-6	Sheath Blight Tolerance	126418290	125999511	18.90	48.34	112679044	98.62	
17	SM-92	Yellow Stem Borer Tolerance	108091210	107729029	16.16	41.33	95315134	98.71	

18	SM-93	Early Maturation and High Yield	131344752	115584547	17.34	44.34	115584547	98.90	

Table 4: Number of EMS-induced homozygous SNPs (GC to AT type), dissimilarity index, and the frequency of SNPs in the mutant lines with respect to SM

S.No.	Line	Trait	Number of SNPs	Dissimilarity index (%)[a]	SNPs per Mb
1	TI-109	Complete Panicle Exsertion	69765	0.018	178.4
2	TI-110	Complete Panicle Exsertion	59320	0.015	151.7
3	TI-128	High Yield; High Spikelets per Panicle	126019	0.032	322.3
4	TI-140	Long Slender Grain	329320	0.084	842.3
5	TI-17	Sheath Blight Tolerance and Strong Culm	326555	0.084	835.2
6	TI-170B	Strong Culm	342527	0.088	876.0
7	TI-26	Strong Culm	348872	0.089	892.3
8	TI-35	Elongated Uppermost Internode	34121	0.009	87.3
9	TI-38	Complete Panicle Exsertion	65556	0.017	167.7
10	TI-42	Bacterial Blight Tolerance	29850	0.008	76.3
11	TI-50	High Yield; High Spikelets per Panicle	124933	0.032	319.5
12	TI-51	Long Slender Grain	334905	0.086	856.5
13	SB-6	Sheath Blight Tolerance	37387	0.010	95.6
14	SM-92	Yellow Stem Borer Tolerance	317964	0.081	813.2
15	SM-93	Early Maturation, High Yield, Yellow Stem Borer tolerance	282113	0.072	721.5

Figures

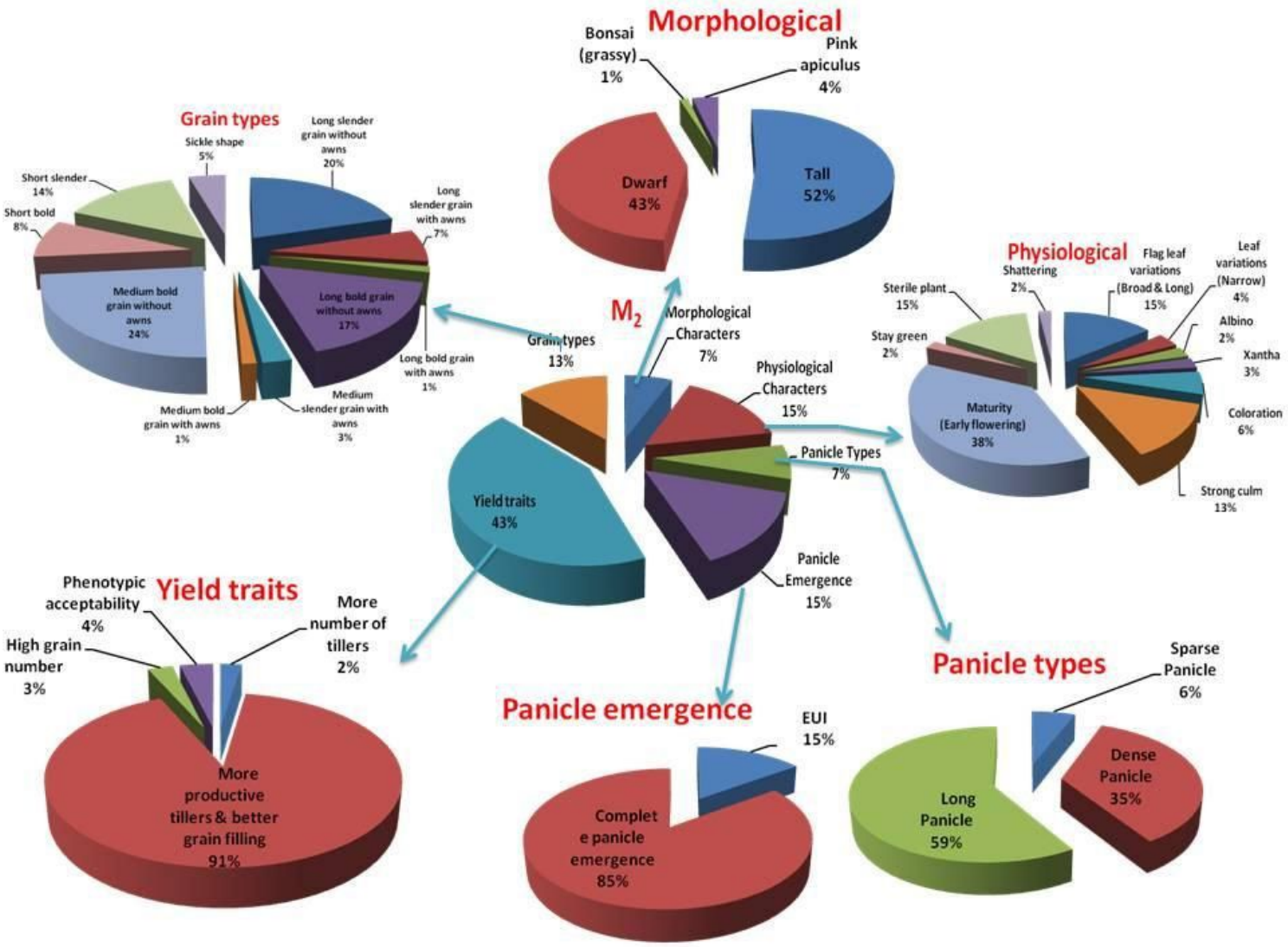

Figure 1

Distribution of mutants for various agro-morphological traits in M2 generation.

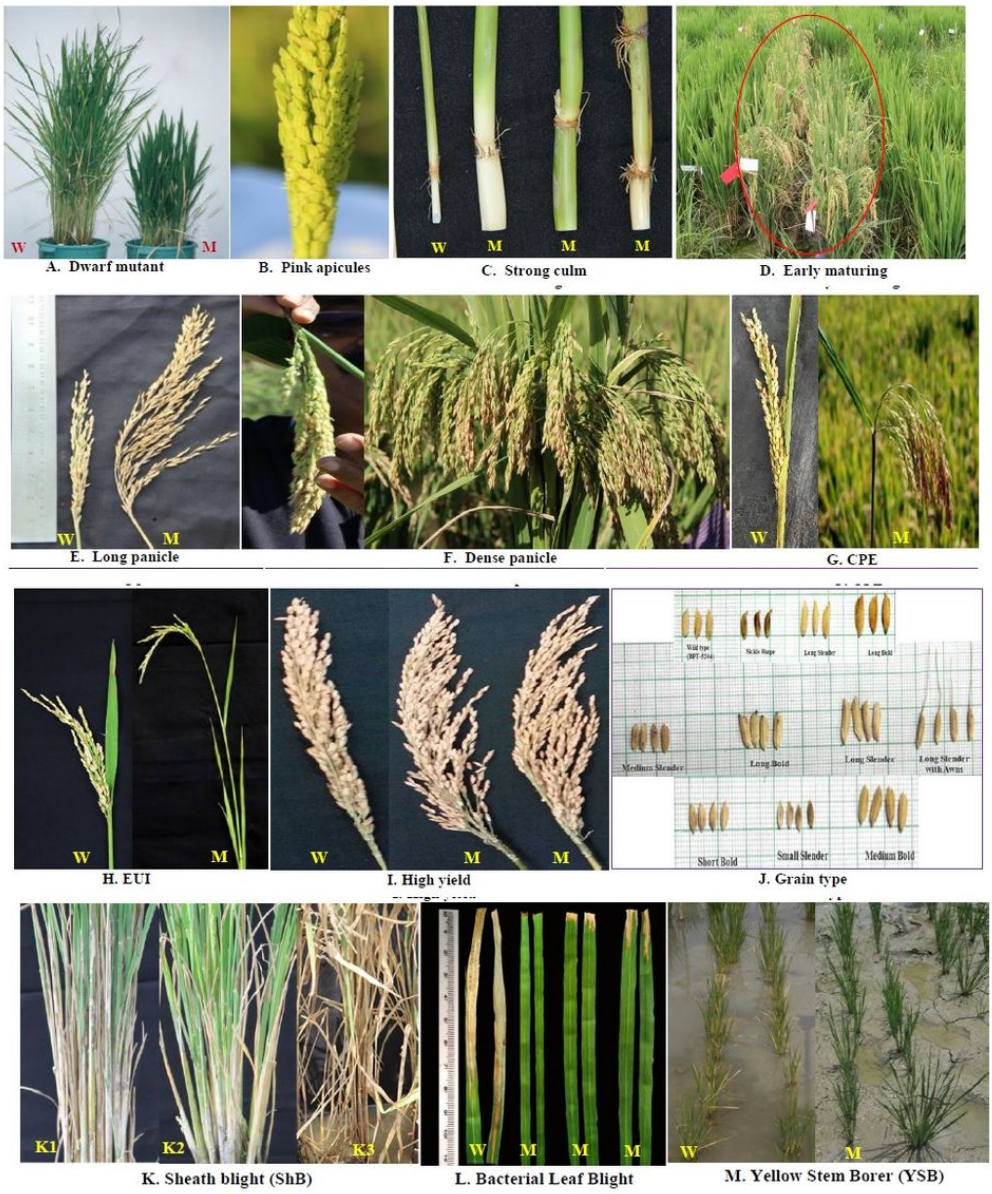

Figure 2

A – M: Variants for different traits in the mutagenized population of Samba Mashuri ; W- wild type Samba Mashuri ; M- Mutant; K1- Wild type with score 9; K2- Resistant mutant (SB 6) with score 1 and K3- Susceptible check TN 1 with score 9.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS13SNPsincodingregion.xlsx
- TableS12GenomicsimilaritySSRmarkers.xls
- TableS11Multipletraitdata.xls
- TableS10BLBdata.xls
- TableS9sheathblightdata.xls
- TableS8YSBdata.xls

- TableS7M3toM5graintypedata.xlsx
- TableS6M3toM5yielddata.xlsx
- TableS5M3toM5Panicleemergencedata.xlsx
- TableS4M3toM5Panicletypedata.xlsx
- TableS3M3toM5Physiologicaldata.xlsx
- TableS2M3toM5Morphologicaldata.xlsx
- TableS1ListofSSRmarkers.xls
- FigureS1.jpg